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Antibiofilm Effects of Chitosan-phytochemical Compounds against Biofilm-forming Food Pathogenic Bacteria

by

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(바이오 필름을 형성하는 식중독 균에 대한 키토산 유도체의 바이오 필름 생성 저해 효과)

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by

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List of Abbreviation

ATCC	**American Type Culure Collection**
BEC	**Biofilm eradication concentraion**
BIC	**Biofilm inhibitory concentration**
CCA	**Chitosan-caffeic acid**
CFA	**Chitosan-ferulic acid**
CSA	**Chitosan-sinapic acid**
EPS	**Extracellular poylmeric substace**
KCCM	**Korean Culture Center of Microorganisms**
KCTC	**Korean Collection for Type Cultures**
MBC	**Minimum bactericidal concentration**
MIC	**Minimum inhibitory concentration**

바이오 필름을 형성하는 식중독 균에 대한 키토산 유도체의 바이오 필름 생성 저해 효과

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요 약

바이오 필름(biofilm)이란 표면에 부착된 미생물 집단을 일컫는 말로 식품, 질병, 의료기기 분야뿐만 아니라 세탁기, 에어컨, 가습기를 포함한 생활가전 그리고 배수구 등 넓고 다양한 분야에서 심각한 문제를 일으킨다. 일단 바이오 필름이 형성되면 그 안의 미생물은 항생물질에 대해 상당히 증가된 저항성을 가지게 되는데, 이러한 특성은 바이오 필름 안에서 느려진 세포의 증식 속도, 미생물이 생성하는 extracellular polymeric substance (EPS)에 의한 항생물질 확산 지연 등의 내재적 요인과 세포간의 플라스미드 교환 등의 외재적 요인으로 그 메커니즘이 연구되고 있다. 본 연구에서는 항균, 항산화 및 항염증 효과를 포함한

생리활성 기능을 가진 chitosan에 caffeic acid, ferulic acid 그리고 sinapic acid와 같은 hydroxycinnamic acids를 conjugate 시킨 chitosan-phytochemical compounds를 이용하여, 바이오 필름을 형성하는 식중독균인 *Pseudomonas aeruginosa, Listeria monocytogenes*와 *Staphylococcus aureus*를 대상으로 바이오 필름 생성 억제 및 저해 효과를 측정하였다.

Minimum inhibitory concentration (MIC) 와 minimum bactericidal concentration (MBC) assay를 통해 일반 부유균에 대한 항균 활성을 측정하였으며, 그 결과 전반적으로 대조구 (unmodified chitosan)보다 합성 유도체인 chitosan-phytochemical compounds가 뛰어난 활성을 나타내었다. 특히 실험에 이용된 식중독 균 중 *L. monocytogenes*에 대해 가장 우수한 항균 활성을 나타내었고, 그 다음으로 *P. aeruginosa*와 *S. aureus* 순이었다. Chitosan-phytochemical compounds 별로는 유사한 수준의 항균 활성이 관찰되었으나 chitosan-caffeic acid conjugate (CFA)에서 조금 더 우수한 항균 효과가 나타났다.

Chitosan-phytochemical compounds의 antibiofilm 활성 측정 실험은 크게 두 가지 경우로 나누어 진행되었다. 형성된 바이오 필름에 대한 chitosan-phytochemical compounds의 바이오 필름 저해 효과를 파악하기 위해 biofilm inhibitory concentration (BIC)와 biofilm eradication

concentration (BEC) assay를 진행하였고, 다음으로 chitosan-phytochemical compounds가 바이오 필름 형성 자체를 얼마나 억제하는지 파악하기 위해 MIC의 농도 보다 낮은 sub-inhibitory concentration(sub-MIC) 수준에서 (0.5 MIC, 0.25 MIC, 0.125 MIC) 형성된 바이오 필름을 염색하는 safranin stain assay를 진행하였다. 동시에 항균 활성과 형성된 바이오 필름에 대한 저해 활성의 비교 분석을 통해, 일반 부유균 대비 바이오 필름이 가지는 항생물질에 대한 증가된 저항성을 파악하였다. Antibiofilm 활성 역시 대조구 대비, chitosan-phytochemical compounds에서 더 우수한 효과가 나타났으며 균의 종류 및 물질에 따라서도 항균 활성과 그 경향이 유사하였다. 또한 형성된 바이오 필름을 저해하는데 요구되는 chitosan-phytochemical compounds의 농도는 일반 부유균을 저해하는데 요구되는 것보다 최소 2배에서 최대 16배 높은 값을 나타내었다.

바이오 필름은 여러 단계를 거쳐 복잡한 과정으로 형성된다. 일단 미생물이 표면에 부착하면 이들은 새로운 환경에 적응하기 위해 quorum sensing을 통하여 여러가지 신호 물질을 생성하고 이에 반응한 미생물은 exopolysaccharide, extracellular DNA, Polypeptide 등 여러가지 물질로 구성된 EPS를 생성한다. 이렇게 생성된 EPS는 바이오 필름의 전반적인

3차원구조와 골격을 구성하며 미생물 집단을 더욱 단단히 한다. Sub-MIC 농도에서 safranin으로 염색된 바이오 필름은 chitosan-phytochemical compounds가 바이오 필름 형성을 얼마나 효과적으로 억제하였는지 나타낸다. 실험 결과, chitosan-phytochemical compounds의 농도가 희석됨에 따라 바이오 필름 형성은 점차 높은 비율로 관찰되었으나 바이오 필름 형성 억제 효과는 키토산 유도체의 종류 및 균 종류마다 각기 다르게 나타났다. 이에 대해서는 균의 종류마다 바이오 필름을 형성하는 구성 성분 및 구조가 다르며 항생물질에 대한 반응 또한 각기 다른 메커니즘으로 이루어지기 때문으로 그 원인을 추정하며 추후 chitosan-phytochemical compounds의 antibiofilm 메커니즘에 대한 연구를 진행할 예정이다.

현재까지 진행된 많은 연구들은 다양한 실험 방법으로 여러 가지 물질의 바이오 필름 억제 효과를 보고하고 있다. 부추, 마늘, 루이보스, 카멜리아 신네시스, 에키나세아, 감초 등의 약용 식물 1,000 μg/mL 농도는 *L. monocytogenes* 의 바이오 필름 형성을 25 ~ 80 % 억제한다고 하며, 또 다른 연구는 키토산이 *S. aureus*의 바이오 필름을 일반적으로 약 60% 정도까지 억제한다고 보고하였다. 이와 비교하였을 시 본 연구에서 이용된 Chitosan-phytochemical compounds은 바이오 필름을 형성하는

식중독 균에 대해 더 우수한 antibiofilm 활성을 보였으며 특히 *L. monocytogenes*에 대해 가장 뛰어난 효과를 나타내었다. 본 연구의 결과를 통해 chitosan-phytochemical compound이 항균, 항염증, 항암 활성뿐만 아니라 antibiofilm 활성을 지닌 천연 유래 기능성 물질로서 바이오 필름을 생성하는 식중독 균 제어에 유용하게 이용될 수 있을 것으로 기대된다.

Introduction

Biofilm, a surface-attached bacterial community, causes serious problems detected in wide fields such as food, disease, medical equipment, drainage and household appliances including air cleaner and washing machine. Therefore, biofilms have considerable influence in a variety of fields and most bacteria have an ability to attach to a surface, leading formation of a biofilms (Van Houdt and Michiels, 2010). In addition, one of the important features of biofilm is increased resistance against several chemical agents including antibiotics and sanitizers in biofilm formed bacteria. It has been known that a major factor causing the high resistance is an extracellular polymeric substance (EPS) composing the biofilm (Lewis K, 2007; Mah et al., 2003). EPS consists of extracellular DNA, polysaccharides, proteins and others (Favre-Bonté et al., 2010; Ganguly et al., 2011; Storz et al., 2012). It builds three dimensional structures that hold bacteria together for cell-cell communication and make it easy to supply nutrients to bacteria (Stoodley et al., 2002).

Pseudomonas aeruginosa, Listeria monocytogenes and *Staphylococcus aureus* are major food pathogenic bacteria to form biofilms (Deza et al., 2005;

Langsrud et al., 2003; Shi and Zhu, 2009). *P. aeruginosa* causing an opportunistic pathogen exhibits a high tendency to form biofilm, and strong resistance against disinfectant commonly used in food industries (Poulsen, 1999). This bacterium attaches to stainless steel and the cells adhered on steel twice the numbers of cells in planktonic status (Stanley, 1983). *L. monocytogenes* and *S. aureus* are also food pathogenic bacteria, which are able to form biofilms on surfaces of glass, stainless steel, rubber and plastics used in food manufacturing plants (Gomes et al., 2012). Biofilm formed by *L. monocytogenes* also causes a serious risk in food processing because they can persist for long periods of time in the food processing environment and thus represent a source of recurrent contamination (Møretrø and Langsrud, 2003). Moreover, *S. aureus* associated with biofilm required 110 times minimum bactericidal concentration (MBC) of vancomycin to provide a 3-log reduction compared to its planktonic cells (Williams et al., 1997).

As described above, biofilms are a major concern in various industry including food processing, medical implants, paper manufacturing, dental plaque and others. For these reasons, many studies have been focused on controlling and eliminating biofilm-forming bacteria using various chemical materials. However, it is well known that treatments with chemical agents have potential side effects. It has been reported that safer and productive forms of cleaning procedures for biofilm removal were being tested in food

processing industries, but these also have a side effect releasing toxic gases into environment (Kumar and Anand, 1998). Therefore, many studies have been conducted on inhibitory effects of natural materials with low toxicity against biofilm-forming bacteria (Fu et al., 2011; Schaschke and Audic, 2014; Tello et al., 2011).

Chitosan is the poly-D-glucosamine derived from chitin, which is a natural polymeric material extracted from crustaceans such as crab and shrimp (Rinaudo, 2006). It possesses superb properties like biocompatibility, biodegradability, low-toxicity and non-immunogenetics, so many researchers have conducted a number of studies in order to determine the potential material in food, agricultural and pharmaceutical industry (Jang and Nah, 2008). Chitosan has a similar structure with cellulose, but there is an amino group at the 2-carbon position, which is important role in antibacterial or antiviral activity (Lee et al., 2012). Not only antibacterial activity but also antibiofilm activity of chitosan has been also evaluated against a variety of bacteria such as food and oral pathogens (Costa et al., 2014; Orgaz et al., 2011). However, chitosan has a serious limiting factor due to its low water-solubility. For this reason, many studies take a profound interest in developing novel chitosan derivatives conjugated with other functional materials. There are several reports on studying antibacterial and antibiofilm activity of chitosan derivatives (Kenawy et al., 2007; Silva et al., 2014).

Hydroxycinnamic acids included in phenolic acid are phytochemicals presenting in many food sources like coffee, apples, cider, blueberry and other many plant source. In addition, these phytochemicals possess several bioactivities including antimicrobial and antioxidant (Lee et al., 2014a; Lee et al., 2014b; Magnani et al., 2014). From this point of view, some researchers previously investigated antibacterial, antioxidant, cytotoxicity and anti-inflammatory effects of chitosan-phytochemical compounds which are composed of chitosan derivative hydroxycinnamic acid such as caffeic acid, ferulic acid and sinapic acid (Lee et al., 2014a; Lee et al., 2014b). However, no further experiment was progressed on antibiofilm effect of chitosan-hydroxycinnamic acid conjugates against biofilm-forming food pathogenic bacteria.

Therefore, the aim of this study was to evaluated an inhibitory efficacy of chitosan--phytochemical compounds (chitosan-caffeic acid, chitosan- ferulic acid and chitosan-sinapic acid) against biofilm-forming food pathogenic bacteria such as *P. aeruginosa, L. monocytogenes* and *S. aureus*. The results obtained in this study will provide valuable information on the development of antibiofilm agents.

Materials and Methods

1. Materials

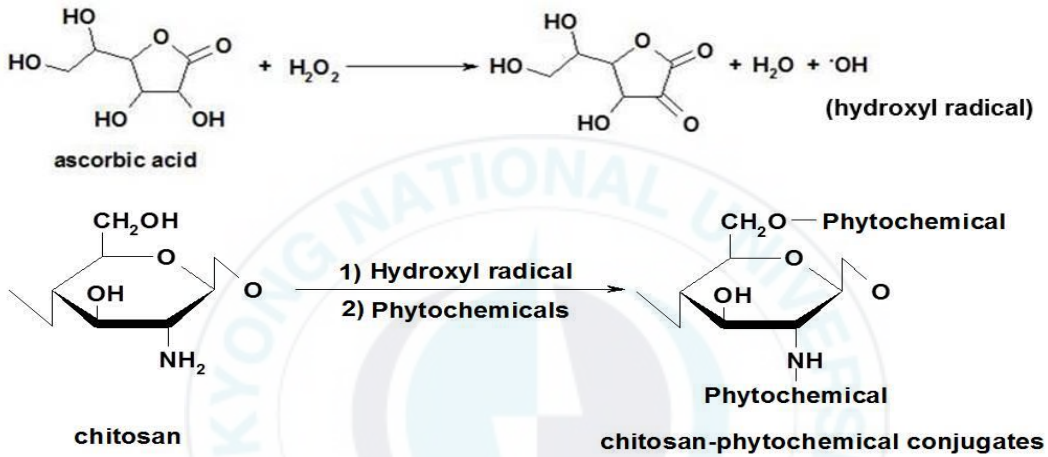
Chitosan (average MW 310 kDa and 90% degree of deacetylation) was purchased from Kitto life Co. (Seoul, Korea). Hydroxycinnamic acids such as caffeic acid, ferulic acid and sinapic acid were also purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade and commercially available.

2. Preparation of chitosan-phytochemical compounds

Chitosan-phytochemical compounds were kindly provided by Prof. Jae-young Je, Pukyong National University. The compounds were prepared according to his previous method, with a minor modification (Cho et al., 2011; Fig. 1). In brief, 0.25 g of chitosan was dissolved in 25 mL of 2% acetic acid, and 0.5 mL of 1.0 M hydrogen peroxide containing 0.054 g of ascorbic acid was then added. After 30 min at room temperature, 0.14 mM

hydroxycinnamic acids (0.02514 g of caffeic acid, 0.02709 g of ferulic acid and 0.03128 g of sinapic aicd) were added to the mixture and allowed to react for 24 h at room temperature. Untreated hydroxycinnamic acid was removed by a dialysis with 1 kDa dialysis tubing (Thermo-Fisher Scientific, Rockford, USA). The resulting chitosan-phytochemical compounds were designated as chitosan-caffeic acid (CCA), chitosan-ferulic acid (CFA) and chitosan-sinapic acid (CSA), respectively (Fig. 1). In addition, the unmodified chitosan was also treated with the same procedures without any hydroxycinnamic acid.

1) Generation of hydroxyl radical and synthesis pathway of chitosan-phytochemical compounds



2) Four kinds of hydroxycinnamic acids

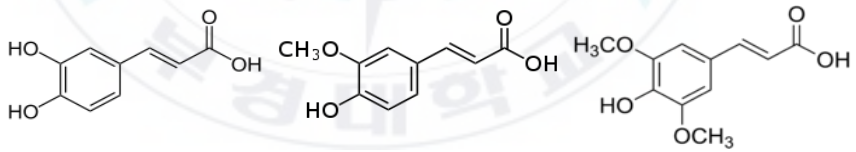


Fig. 1. Synthesis pathway of chitosan-phytochemical compounds (source : Kang, 2015; Kim, 2016)

3. Bacterial strains and medium

P. aeruginosa standard bacterial strain KCCM 11321 (ATCC 15442) was obtained from the Korean Culture Center of Microorganisms (KCCM; Seoul, Korea) to assure reliability of research results. *L. monocytogenes* standard strains KCTC 3569 (ATCC 19111) and *S. aureus* KCTC 1916 (ATCC 6538) were purchased from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). Other isolated bacteria, three of *L. monocytogenes* strains and six of *P. aeruginosa* strains were provided from Gyeoingsang national university hospital. These strains were grown aerobically at 37℃ in in tryptic Soy Broth (TSB; Difco Labotatory Inc., Detroit, MI, USA) and were subsequently used in experiments to measure antibacterial and antibiofilm activity.

Table 1. Bacterial strains used for antibacterial activity

Strains	Strain sources
Pseudomonas aeruginosa	**KCCM 11321 (ATCC 15442)**
Listeria monocytogenes	**KCTC 3569 (ATCC 19111)**
Staphylococcus aureus	**KCTC 1916 (ATCC 6538)**
***P. aeruginosa* isolate 48**	**Gyeongsang National University Hospital**
***P. aeruginosa* isolate 152**	
***P. aeruginosa* isolate 366**	
***P. aeruginosa* isolate 1842**	
***P. aeruginosa* isolate 2179**	
***P. aeruginosa* isolate 3248**	
***L. monocytogenes* isolate 2148**	**Gyeongsang National University Hospital**
***L. monocytogenes* isolate 2637**	
***L. monocytogenes* isolate 2868**	

4. A quantitative antibacterial assay to planktonic bacterial cells

The two-fold serial dilution method with tryptic soy broth (TBS) was used for determination of minimum inhibitory concentration (MIC) and MBC of chitosan-phytochemical compounds against *P. aeruginosa, L. monocytogenes* and *S. aureus* (NCCLS, 2003). MIC was defined as the lowest concentration of crude extract that inhibited the visual growth after incubation at 37℃ for 20-24 h and was performed in triplicates (Grierson and Afolayan, 1999). The minimum concentration of chitosan-phytochemical compounds that reduces bacterial numbers by at least 3 logs was defined as MBC (Amyes et al., 1996).

5. A quantitative assay for antibiofilm activity

Inhibitory effect of chitosan-phytochemical compounds on established biofilm was verified by the method of Johnson et al. (2002) with slight modifications. Biofilms of all strains were formed on bottom of microtiter plates. The planktonic bacteria were removed after incubation for 24 h at 37℃. The wells were washed three times with phosphate buffer saline (PBS; 0.1M, pH 7.4)) and filled with 200 mL twofold dilutions of the chitosan-

phytochemical compounds. The plates were incubated for 24 h at 37℃. The OD_{492} was measured two times at 0 h and after incubation for 24 h. The biofilm inhibitory concentration (BIC) values were determined as the lowest concentration at which no increase in optical density compared with the 0 h OD_{492}. Biofilms in the bottom of plate wells were scarified by a loop and spread over the surface of tryptic soy agar (TSA; Difco Labotatory Inc.) plates, then incubated for 72 h at 37℃. The biofilm eradication concentration (BEC) values were determined as the lowest concentration at which no bacteria were grown on the TSA plates.

6. Safranin staining assay for determining biofilm formation

The effect of different concentrations of chitosan-phytochemical compounds on biofilm formation was investigated on microtiter plates (Cramton et al., 1999). All strains were grown for 24 h in 10 mL TSB with 1% glucose, diluted in growth medium to 5×10^5 CFU/mL and 100 mL was dispensed into each well of microtiter plates in the presence of 100 mL sub-inhibitory concentrations (sub-MIC) of chitosan-phytochemical compounds (0.5, 0.25 and 0.125 MIC) and 100 ml medium (control). After incubation for 24 h at

37°C, each well was washed twice with PBS, dried, stained with 0.1% safranin for 1 min and washed with water. The stained biofilms were resuspended in 200 mL PBS and the cell suspended solution was measured at OD_{492} using an ELISA reader (GENios® microplate reader; Tecan Austria GmbH; Grödig, Austria) .

Results and Discussion

1. Antibacterial efficacy of chitosan-phytochemical compounds on planktonic bacterial cells

The present study focused on an antibiofilm activity of chitosan-phytochemical compounds against biofilm-forming food pathogenic bacteria such as *P. aeruginosa, L. monocytogenes* and *S. aureus*. Therefore, it is another important factor to evaluate antibacterial effects of chitosan-phytochemical compounds in order to compare inhibitory effects on planktonic bacteria with those on biofilm. The MIC and MBC values of the chitosan-phytochemical compounds were determined by the two-fold serial dilution method, and the results are summarized in Table 2 and 3. The MIC values of the unmodified chitosan were lower than those of chitosan-phytochemical compounds against all experiment bacteria strains. In this study, the MIC values of chitosan-phytochemical compounds were observed similarly against all tested bacteria. Furthermore, the MBCs, which were showed higher values than those of the MICs against bacteria, indicated that the chitosan-phytochemical compounds have higher antibacterial activity than

those of unmodified chitosan. Interestingly, chitosan-phytochemical compounds showed the best inhibitory effect against *L. monocytogenes* and followed by *P. aeruginosa* and *S. aureus*.

As described above, unmodified chitosan possesses strong antibacterial effect against *L. monocytogenes*. Interestingly, the activity of unmodified chitosan exhibiting MICs of 64-512 μg/mL were enhanced by the conjugation with hydroxycinnamic acids even though the phytochemical exhibited poor antibacterial activity against *L. monocytogenes* ranging in 2,000-2,500 μg/mL (Wen et al., 2003). Lee et al. (2014b) previously reported that MICs of chitosan-phytochemical compounds against *L. monocytogenes* were ranging in 32-64 μg/mL. The results obtained in this study also similar with the previous results, demonstrating that chitosan-phytochemical compounds possess a higher antibacterial effect against food pathogenic bacteria.

In addition, the MIC value of chitosan against *L. monocytogenes* was reported from 150 to 800 μg/mL (Goy et al., 2009), which was higher MICs than the results in this paper. As well known, it is difficult to compare antibacterial effect of chitosans and its derivatives because of possible differences in (1) characteristics (de-acetylation and polymerization degree) of the chitosan used in various experiments (2) experimental temperature and pH, and (3) chitosan solvent, organic acids being better than inorganic acids and organic solvents with higher carbon numbers having decreased

antimicrobial activity (Chung et al., 2003). For these reasons, there will be a little difference between the results provided different research groups on antibacterial activity of the chitosans and its derivatives. However, it is obvious that chitosan derivatives exhibit higher antibacterial effects on pathogenic bacteria than the unmodified chitosan as like reported in this study (Lee et al., 2014a; Lee et al., 2014b).

Antibacterial mechanism of chitosan depends on its de-acetylation and amino group (Goy et al., 2009). Positively charged chitosan interacts with the negatively charged bacterial cell surface, leading to weakening of the cell wall either by cell wall damage alone or accompanied by cell lysis (Eaton et al. 2008; Lee et al., 2014a). It has been known that the antibacterial mechanism of hydroxycinnamic acids is like as phenolic compounds do. In detail, phenolic compounds affect enzyme activity related to energy production of bacteria at low concentrations, but they cause protein denaturation of bacteria at high concentrations (Bajpai et al., 2008; Fung et al., 1977; Rico-Munoz et al., 1987). Moreover, the affinity of chitosan for extracellular membrane of bacteria is increased by chitosan-phytochemical compounds because hydroxycinnamic acids possess unsaturated chain (Lee et al., 2014a; Sánchez-Maldonado et al. 2011). Thus, the antibacterial activities of chitosan-phytochemical compounds were higher than that of unmodified chitosan (Lee et al., 2014a; Lee et al., 2014b).

Table 2. Minimum inhibitory concentrations (MIC) of the chitosan-phytochemical compounds against food pathogenic bacteria

Strain	MIC (μg/mL)			
	CCA[a]	CFA	CSA	Unmodified chitosan
P. aeruginosa (KCCM 11321)	512	512	1,024	1,024
P. aeruginosa isolate 48	512	512	1,024	2,048
P. aeruginosa isolate 152	256	256	256	1,024
P. aeruginosa isolate 366	128	128	128	2,048
P. aeruginosa isolate 1842	128	128	128	1,024
P. aeruginosa isolate 2179	256	512	512	2,048
P. aeruginosa isolate 3248	128	128	256	1,024
L. monocytogenes (KCTC 3569)	64	64	128	64
L. monocytogenes isolate 2148	128	128	128	512
L. monocytogenes isolate 2637	32	32	64	128
L. monocytogenes isolate 2868	32	32	64	64
S. aureus (KCTC 1916)	512	512	512	2,048

[a]CCA, chitosan-caffeic acid; CFA, chitosan-ferulic acid and CSA, chitosan-sinapic acid

Table 3. Minimum bactericidal concentrations (MBC) of the chitosan-phytochemical compounds against food pathogenic bacteria

Strain	MBC (μg/mL)			
	CCA[a]	CFA	CSA	Unmodified chitosan
P. aeruginosa (KCCM 11321)	1,024	512	1,024	2,048
P. aeruginosa isolate 48	1,024	1,024	1,024	2,048
P. aeruginosa isolate 152	512	512	512	2,048
P. aeruginosa isolate 366	256	256	256	4,096
P. aeruginosa isolate 1842	128	128	128	1,024
P. aeruginosa isolate 2179	256	512	512	2,048
P. aeruginosa isolate 3248	128	128	512	1,024
L. monocytogenes (KCTC 3569)	128	128	256	128
L. monocytogenes isolate 2148	128	256	256	512
L. monocytogenes isolate 2637	32	64	64	128
L. monocytogenes isolate 2868	32	32	64	128
S. aureus (KCTC 1916)	512	512	512	2,048

[a]CCA, chitosan-caffeic acid; CFA, chitosan-ferulic acid and CSA, chitosan-sinapic acid

2. Inhibitory effect of chitosan-phytochemical compounds on established biofilm

A biofilm that has high persistence in removal and high resistance by the treatment of disinfectants can be grown on food contact surface, resulting in contamination which causes food spoilage and food borne diseases (Van and Michiels, 2010). Chitosan-phytochemical compounds were demonstrated strong antibacterial activity against biofilm forming pathogenic bacteria in the condition of planktonic cells (Table 2 and 3). An antibiofilm effect of chitosan-phytochemical compounds was quantitatively evaluated using BIC and BEC assay.

The BIC and BEC values of the chitosan-phytochemical compounds were investigated by the two-fold serial dilution method using polystyrene flat-bottomed microtiter plate, and the BIC and BEC values are summarized in Table 4 and 5. The BIC values of the unmodified chitosan were observed in range of 2,048 to 16,384 μg/mL for all bacteria strains used in this experiment, which is higher than values of chitosan-phytochemical compounds in both BIC and BEC values. In particularly, CFA showed the most superb inhibitory effects on *L. monocytogenes* biofilm, but there is little different antibiofilm activity within chitosan-phytochemical compounds including CCA, CFA and

CSA. As expected, the BECs which mean biofilm eradication concentration values were higher than the BICs.

Interestingly, BIC values were up to 16 times higher than MIC values, which mean that removal of biofilm cells require much higher concentration of antibacterial agents or antibiotics compared to planktonic cells. In addition, BEC values were also up to 16 times higher than that of BIC values while MBC values were just up to two times higher than that of MIC values. These results signified that the biofilm was once formed, it is very hard to eliminate completely due to the increased resistance. There were many reports as similar with the results obtained in this study. Antonia et al. (2007) reported that BICs of essential oils against *S. aureus* and *S. epidermids* biofilm were increased up to 4 times than that of MICs. It has been also reported that BIC values of antibiotics including ceftazidime. tobramycin, ciprofloxacin, doripenem, piperacillin and colistin against *P. aeruginosa* were 40-1,280 times higher than MIC values (Dosler S and Karaaslan E, 2014).

Table 4. Biofilm inhibitory concentrations (BIC) of the chitosan-phytochemical compounds against food pathogenic bacteria

Strain	BIC (μg/mL)			
	CCA[a]	CFA	CSA	Unmodified chitosan
P. aeruginosa (KCCM 11321)	2,048	2,048	4,096	16,384
P. aeruginosa isolate 48	512	512	1,024	4,096
P. aeruginosa isolate 152	512	512	512	2,048
P. aeruginosa isolate 366	1,024	512	256	2,048
P. aeruginosa isolate 1842	512	512	512	1,024
P. aeruginosa isolate 2179	512	1,024	1,024	4,096
P. aeruginosa isolate 3248	1,024	1,024	1,024	16,384
L. monocytogenes (KCTC 3569)	1,024	1,024	1,024	16,384
L. monocytogenes isolate 2148	2,048	512	1,024	4,096
L. monocytogenes isolate 2637	512	128	512	2,048
L. monocytogenes isolate 2868	512	512	512	2,048
S. aureus (KCTC 1916)	4,096	4,096	4,096	16,384

[a]CCA, chitosan-caffeic acid; CFA, chitosan-ferulic acid and CSA, chitosan-sinapic acid

Table 5. Biofilm eradication concentrations (BEC) of the chitosan-phytochemical compounds against food pathogenic bacteria

Strain	BEC (μg/mL)			
	CCA[a]	CFA	CSA	Unmodified chitosan
P. aeruginosa (KCCM 11321)	4,096	4,096	2,048	16,384
P. aeruginosa isolate 48	2,048	2,048	4,096	16,384
P. aeruginosa isolate 152	2,048	2,048	2,048	16,384
P. aeruginosa isolate 366	4,096	4,096	4,096	32,768
P. aeruginosa isolate 1842	8,192	8,192	4,096	16,384
P. aeruginosa isolate 2179	4,096	4,096	4,096	32,768
P. aeruginosa isolate 3248	2,048	2,048	8,192	65,536
L. monocytogenes (KCTC 3569)	2,048	2,048	2,048	32,768
L. monocytogenes isolate 2148	2,048	2,048	2,048	16,384
L. monocytogenes isolate 2637	2,048	2,048	1,024	32,768
L. monocytogenes isolate 2868	1,024	1,024	2,048	16,384
S. aureus (KCTC 1916)	8,192	8,192	4,096	16,384

[a]CCA, chitosan-caffeic acid; CFA, chitosan-ferulic acid and CSA, chitosan-sinapic acid

3. Inhibitory effect of chitosan-phytochemical compounds on biofilm formation

In order to study further on antibiofilm activity of chitosan-phytochemical compounds, an inhibitory effect on biofilm formation, not established biofilm, was also investigated using biofilm stain method with 0.1% safranin on the levels of sub-inhibitory concentrations. Although chitosan-phytochemical compounds exhibited a different inhibitory effect by each strain, a general attenuated level of biofilm formation according to sub-MIC (0.5 MIC, 0.25 MIC and 0.125 MIC) of CCA, CFA and CSA was observed. Meanwhile, there is no constant pattern of inhibitory effect on biofilm formation by each strain and chitosan-phytochemical compounds. The reason is that many components including polysaccharides, poly-proteins and extracellular DNA and other factors are associated with biofilm feature. As the results, each bacterium has different biofilm features. For example, polysaccharides such as alginate, *Pel* and *Psl* are important comportment of *P. aeruginosa* biofilm determining for the structure of biofilm and its biofilm architecture is mushroom-like structure (Ryder et al., 2007; Banin et al., 2006). However, *L. monocytogenes* has a network of knitted chains biofilm structure which composed with poly-(1,4)-N-acetylamannosamine, BapL, InlA and

FlaA, etc (Köseoğlu et al., 2015; Jordan et al., 2008; Rieu et al., 2008). In case of *S. aureus*, this bacterium produces poly-(1,6)-N-acetyl-D-glucosamine (PNAG) which is a polysaccharide intercellular adhesion (PIA) as a surface polysaccharide (Cramton et al., 1999).

It has been reported that 1 mg/mL of medicinal plant extracts (*Agathosma betulina, Allium sativum, Aspalathus linearis, Camellia sinensis, Echinacea angustifolia, Glycyrrhiza glabra, Hypericum perforatum, Leptospermum petersonii, Melaleuca alternifolia, Mentha piperita, Rosmarinus officinalis* and *Syzygium aromaticum*) showed antibiofilm activity against *L. monocytogenes* from 25% to 80%, while 32-64 μg/mL of chitosan-phytochemical compounds inhibited 52.0-80.1% of biofilm formation by *L. monocytogenes*, which is even more effective (Sandasi amd Vilijeon, 2010).

From all results obtained in this study, it is investigated that chitosan phytochemical compounds are effective on inhibition of biofilm-forming bacteria, especially *L. monocytogenes*. However, mechanism of antibiofilm activity of chitosan-phytochemical compounds against biofilm-forming bacteria remains unknown. In order to study in more detail to elucidate the antibiofilm mechanism of chitosan-phytochemical compounds against these bacteria, it is necessary to investigate an inhibitory effect on transcriptional regulation of genes associated with biofilm formation or on disruption of protein expression.

Therefore, RNA isolation and real-time quantitative PCR (RE-PCR) will be conducted focusing on *L. monocytogenes* which exhibited the most superb inhibitory effect in both antibacterial and antibiofilm. As listed in Table 10, there is a study that suggests that expression of *L. monocytogenes* genes critical for biofilm synthesis *(flaA, fliP, fliG, flgE, motA, motB, prfA, degU, mogR, dnaK, agrA, agrB, agrC)* was investigated using RT-qPCR. In detail, *flaA, fliP, fliG, flgE, motA,* and *motB* play a role for initial attachment of biofilm formation while *agrA, agrB* and *agrC* function as quorum sensing. *dnaK* acts for stress response and *prfA, degU* and *mogR* do transcriptional regulation (Ollinger et al., 2009). Based on this information, it will need to conduct a study on inhibitory effect of chitosan-phytochemical compounds on expression gene or proteins associated with biofilm forming in molecular level using RT-PCR or western blot analysis.

Table 6. Inhibitory effect of chitosan-phytochemical compounds on biofilm formation 1

Strains	Samples	Biofilm formation value[a]		
		0.5 MIC	0.25 MIC	0.125 MIC
P. aeruginosa ATCC 15442	Unmodified[b]	32.358	51.246	86.929
	CCA	38.804	40.886	52.740
	CFA	36.706	79.148	86.167
	CSA	37.797	72.571	88.047
P. aeruginosa isolate 48	Unmodified[b]	49.483	69.040	107.739
	CCA	40.710	83.870	116.270
	CFA	30.329	55.453	90.141
	CSA	43.861	53.867	115.838
P. aeruginosa isolate 152	Unmodified[b]	43.080	44.190	75.244
	CCA	28.050	32.090	47.408
	CFA	25.423	56.564	67.435
	CSA	34.901	58.386	87.027
P. aeruginosa isolate 366	Unmodified[b]	43.923	84.438	107.079
	CCA	31.051	79.508	100.935
	CFA	30.454	93.036	97.530
	CSA	32.498	61.424	122.985

[a]Biofilm formation values were calculated as: (mean OD_{492} treated well)/(mean OD_{492} control well×100.

[b]Unmodified, unmodified chitosan; CCA, chitosan-caffeic acid; CFA, chitosan-ferulic acid and CSA, chitosan-sinapic acid

Table 7. Inhibitory effect of chitosan-phytochemical compounds on biofilm formation 2

Strains	Samples	Biofilm formation value[a]		
		0.5 MIC	0.25 MIC	0.125 MIC
P. aeruginosa isolate 1842	Unmodified[b]	45.889	81.232	117.010
	CCA	87.873	96.245	111.706
	CFA	76.044	94.901	105.412
	CSA	69.777	94.867	96.510
P. aeruginosa isolate 2179	Unmodified[b]	86.469	103.987	129.767
	CCA	75.533	92.867	101.891
	CFA	74.530	116.525	140.571
	CSA	59.287	70.034	93.886
P. aeruginosa isolate 3248	Unmodified[b]	73.427	79.348	104.507
	CCA	56.538	81.623	118.771
	CFA	62.700	109.247	120.878
	CSA	47.502	72.989	74.273
L. monocytogenes ATCC 19111	Unmodified[b]	47.973	72.781	105.084
	CCA	25.269	80.173	96.614
	CFA	19.933	23.305	49.756
	CSA	22.939	60.134	83.406

[a]Biofilm formation values were calculated as: (mean OD_{492} treated well)/(mean OD_{492} control well×100.

[b]Unmodified, unmodified chitosan; CCA, chitosan-caffeic acid; CFA, chitosan-ferulic acid and CSA, chitosan-sinapic acid

Table 8. Inhibitory effect of chitosan-phytochemical compounds on biofilm formation 3

Strains	Samples	Biofilm formation value[a]		
		0.5 MIC	0.25 MIC	0.125 MIC
L. monocytogenes isolate 2148	Unmodified[b]	46.420	70.550	105.298
	CCA	51.143	81.903	99.516
	CFA	45.897	50.688	89.599
	CSA	45.083	63.568	99.775
L. monocytogenes isolate 2637	Unmodified[b]	64.847	78.401	85.492
	CCA	75.017	83.614	96.518
	CFA	61.060	84.046	111.282
	CSA	67.227	67.001	96.144
L. monocytogenes isolate 2868	Unmodified[b]	86.469	103.987	129.767
	CCA	75.533	92.867	101.891
	CFA	74.530	116.525	140.571
	CSA	59.287	70.034	93.886
S. aureus ATCC 6538	Unmodified[b]	85.661	98.340	118.073
	CCA	77.600	84.256	151.093
	CFA	69.979	102.273	147.788
	CSA	51.430	98.141	197.793

[a]Biofilm formation values were calculated as: (mean OD_{492} treated well)/(mean OD_{492} control well×100.
[b]Unmodified, unmodified chitosan; CCA, chitosan-caffeic acid; CFA, chitosan-ferulic acid and CSA, chitosan-sinapic acid

Table 9. Functions of *Listeria monocytogenes* genes associated with biofilm forming

Gene	Function
flaA	**Structural flagella protein**
flip	**Flagellar biosynthesis protein**
fliG	**Flagellar motor switch protein**
flgE	**Flagellar hook protein**
motA	**Flagellar motor protein**
motB	**Flagellar motor protein**
prfA	**Transcriptional regulator**
degU	**Transcriptional regulator/quorum sensing**
mogR	**Transcriptional regulator for motility**
dnaK	**Molecular chaperon involve in biofilm**
agrA	**Quorum sensing**
agrB	**Quorum sensing**
agrC	**Quorum sensing**

Conclusion

Biofilm is a sessile microbial matrix adhered to a surface. Once biofilm is formed, bacteria in this slime are hard to eliminate because of its increased resistance. The aim of this study is to evaluate inhibitory effects of chitosan-phytochemical compounds against biofilm-forming bacteria such as *P. aeruginosa, L. monocytogenes* and *S. aureus*. Inhibitory efficacy of chitosan-phytochemical compounds on biofilm was evaluated by MIC and MBC for planktonic bacterial cells and BIC and BEC for biofilm cells. In addition, safranin stain assay for biofilm formation on the sub-inhibitory concentration (0.5, 0.25, 0.125 MIC) was also determined to evaluate inhibitory effect of chitosan-phytochemical compounds on biofilm formation.

In conclusion, chitosan-phytochemical compounds showed the most superb efficacy on *L. monocytogenes*, followed by *P. aeruginosa* and *S. aureus* in both antibacterial and antibiofilm activities. It was investigated that 2-16 times higher concentration of chitosan-phytochemical compounds required for inhibition of biofilm cells than that of planktonic bacterial cells when comparing MICs with BICs. This means that the bacteria have had much higher resistance to antibacterial agents and antibiotics when they formed the biofilms. In addition, it was also investigated that once the biofilm has formed,

the bacteria within it are considerably difficult to eliminate. BEC values were 2-16 times higher than BIC values while MBC values were similar or 2 times higher than MIC values. Considering above discussion, chitosan-phytochemical compounds possess high antibiofilm activity against biofilm-forming bacteria such as *P. aeruginosa, L. monocytogenes* and *S. aureus*. Thus, the results obtained in this study suggest that chitosan-phytochemical compounds have possibility as antibiofilm agents for controlling biofilm-forming food pathogenic bacteria.

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